

# **Reciprocal regulation of GAS5 lncRNA levels and mTOR inhibitor action in prostate cancer cells.**

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**Running title:** mTOR inhibitors and GAS5

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## Synopsis

Levels of GAS5 lncRNA, an apoptosis promoter and riborepressor of the androgen receptor, are down-regulated as prostate cancer cells acquire castrate-resistance, and consequently this molecule may offer new opportunities for the therapy of castrate-resistant prostate cancer. The mTOR pathway regulates GAS5 lncRNA levels, and here we have investigated using mTOR inhibitors to enhance GAS5 lncRNA in preclinical models of prostate cancer. mTOR inhibitors enhanced GAS5 transcript levels in androgen-sensitive but not in androgen-independent cell lines, which exhibit especially low levels of endogenous GAS5 lncRNA. Indeed, in prostate cancer cells, GAS5 silencing induces resistance to, and ectopic GAS expression confers sensitivity to, the cytostatic effects of mTOR inhibitors, thereby demonstrating a role for GAS5 lncRNA in mTOR inhibitor action.

## Abstract

**Background:** New therapies are required for castrate-resistant prostate cancer (CRPC), and growth-arrest specific 5 (GAS5) lncRNA, which riborepresses androgen receptor action, may offer novel opportunities in this regard. This lncRNA promotes the apoptosis of prostate cancer cells and its levels decline as prostate cancer cells acquire castrate-resistance, so that enhancing GAS5 expression may improve the effectiveness of chemotherapies. Since GAS5 is a member of the 5' terminal oligopyrimidine gene family, we have examined mTOR inhibition as a strategy to increase GAS5 expression. Furthermore, we have determined if GAS5 itself mediates the action of mTOR inhibitors, as demonstrated for other chemotherapeutic agents in prostate cancer cells.

**Methods:** The effects of mTOR inhibitors on GAS5 lncRNA levels and cell growth were determined in a range of prostate cancer cell lines. Transfection of cells with GAS5 siRNAs and plasmid constructs was performed to determine the involvement of GAS5 lncRNA in mTOR inhibitor action.

**Results:** First generation mTORC1, combined mTORC1/mTORC2 and dual PI3K/mTOR inhibitors all increased cellular GAS5 levels and inhibited culture growth in androgen-dependent (LNCaP) and androgen-sensitive (22Rv1) cell lines, but not in androgen-independent (PC-3 and DU 145) cell lines. The latter exhibited low endogenous GAS5 expression, and GAS5 silencing in LNCaP and 22Rv1 cells decreased the sensitivity to mTOR inhibitors, whereas transfection of GAS5 lncRNA sensitized PC-3 and DU 145 cells to these agents.

**Conclusion:** mTOR inhibition enhances GAS5 transcript levels in certain prostate cancer cell lines. This selectivity is likely to be related to endogenous GAS5 expression levels, since GAS5 lncRNA is itself required for mTOR inhibitor action in prostate cancer cells.

**Keywords:** Androgen, castrate-resistance, non-coding RNA, apoptosis, rapalogue, chemotherapy

## Introduction

Prostate cancer (PCa) is the second most common malignancy and a leading cause of cancer related mortality in men [1]. Androgen plays a pivotal role in prostate cell biology, with the normal balance between the proliferation and death of glandular epithelial cells being predominantly under the control of androgen [2, 3]. Thus prostate cells require androgen to promote cell survival and proliferation, whereas androgen deprivation induces apoptosis [4, 5]. Androgen deprivation therapy (ADT) is the mainstay for the treatment of advanced prostate cancer, however it is only palliative, as prolonged ADT invariably gives way to castrate-resistant prostate cancer (CRPC) [6] i.e, persistent tumour growth despite castrate levels of serum testosterone [5, 7]. At the cellular level, the development of CRPC represents a major compensatory response to androgen deprivation-induced stress, allowing cancer cells to survive and subsequently thrive in a low testosterone environment [8].

The majority of chemotherapies act via the induction of apoptosis and, currently, there is a critical need to develop new treatments for CRPC, since apoptotic evasion is a key feature of this disease [5, 9, 10]. In this regard, long non-coding RNAs (lncRNAs) may offer new drug targets, as they are increasingly recognised as key regulators of cellular processes and are recognised as of importance in the pathogenesis of many cancers including that of PCa [11-14].

The lncRNA and small nucleolar RNA (snoRNA) host gene, growth arrest-specific 5 (GAS5), is encoded at a prostate cancer-associated locus, 1q25 [15]. The gene was identified via subtractive cDNA cloning for potential novel tumour suppressor genes associated with growth-arrested cells [16]. GAS5 comprises 12 exons which encode two mature lncRNAs [16], while ten box C/D snoRNA are encoded

within introns [17]; three of these snoRNAs (U44, U74 and U78) may also give rise to miRNAs [18]. GAS5 is a member of the 5'-terminal oligopyrimidine tract (5'-TOP) gene family [10, 16, 18]. GAS5 transcripts accumulate in growth arrested cells [14], due to interplay between two pathways [16, 18]. Firstly, the mTOR pathway, through its regulation of translation of RNAs carrying the 5'-TOP sequence and, secondly, nonsense mediated decay (NMD, which degrades transcripts containing stop codons in early exons), due to the short GAS5 reading frame which terminates with a stop codon within exon 3 (of 12) [16, 19]. Thus, in actively growing cells, where mTOR activity is high, translation of the short reading frame is promoted, and degradation of transcripts by the NMD pathway results in low cellular GAS5 levels. Suppression of mTOR activity, such as occurs upon growth arrest, prevents active translation of GAS5 transcripts and their consequent degradation by NMD, resulting in the accumulation of GAS5 transcripts [20, 21].

GAS5 is of a particular interest in CRPC, as its levels are down-regulated in LNCaP xenografts upon the acquisition of castrate-resistance [22]. This is of significance, as a portion of GAS5 sequence mimics the glucocorticoid receptor response element (GRE) and riborepresses glucocorticoid receptor action [23]. More importantly, in the prostate context, GAS5 lncRNA also sequesters the androgen/androgen receptor complex and prevents its binding to target DNA sequences [24]. Moreover, functional studies have shown that GAS5 lncRNA promotes the apoptosis of prostate cancer cells [10], and have demonstrated a quantitative relationship between the extent of cell death and cellular GAS5 lncRNA levels [10]. Enhancement of cellular GAS5 lncRNA levels in combination with chemotherapeutic drugs may therefore offer an improved strategy for the treatment of advanced PCa.

One way to enhance cellular GAS5 lncRNA levels in prostate cancer cells may be through mTOR inhibition, as previously demonstrated for lymphoid and certain breast cancer cell lines [20, 21].

Indeed, there is currently much interest in the clinical use of mTOR inhibitors, either alone or in combination with other therapies, for the treatment of PCa, particularly since activation of the PI3K/Akt/mTOR pathway has been implicated in prostate cancer progression and the development of drug and radiation resistance. Here we have investigated the efficacy of various classes of mTOR inhibitors, comprising allosteric mTORC1, combined mTORC1/mTORC2 and dual PI3K/mTOR inhibitors, in enhancing GAS5 lncRNA levels in a range of prostate cancer cell lines. In addition, we have also examined whether GAS5 lncRNA is itself required for mTOR inhibitor action in prostate cancer cells [10], as has been previously shown for lymphoid cells [20], since this may impact upon the clinical effectiveness of such agents in suppressing cancer growth through the induction of cell cycle arrest and apoptosis.

## **Materials and Methods**

### **Materials**

The prostate cancer cell lines 22Rv1, LNCaP, PC-3 and DU 145 were from ATCC-LGC Promochem (Teddington, UK); PNT2C2 cells were obtained from Prof N. J Maitland (University of York, Heslington, UK). Cell culture materials and classical chemotherapeutic drugs were from Sigma-Aldrich Company Ltd (Gillingham, UK). Sources of other drugs were: rapamycin (Millipore, Watford, UK), everolimus and temsirolimus (LC Laboratories, Woburn, MA), BEZ235 and AZD8055 (Strattech Scientific, Newmarket, UK). TRIsure and Silencer Select siRNAs were from Life Technologies Ltd (Paisley, UK). RNase-free DNase, reverse transcriptase and CellTiter 96 Aqueous One Solution Cell Proliferation Assay was from Promega (Southampton, UK). Brilliant III SYBR Green was from Stratagene (Santa Clara, CA). HiPerFect transfection reagent was from Qiagen (Crawley, UK) and nucleofector solutions were from Lonza Biosciences (Verviers, Belgium).

## **Cell Culture**

LNCaP, PNT2C2 and 22Rv1 were routinely cultured in RPMI-1640, supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 10% fetal bovine serum and 50 µg/mL gentamicin. Culture medium for PC-3 was nutrient mix F-12 (Kaighn's modification) supplemented with 10% fetal bovine serum and 50 µg/mL gentamicin (Sigma). Culture medium for DU 145 was Eagles minimum essential medium (EMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate and 50 µg/mL gentamicin. All cell lines were cultured at 37 °C in a humidified incubator with 5% CO<sub>2</sub>.

## **Plasmid DNA Transfection**

Plasmids were pcDNA3/GAS5.AE (encodes mature GAS5 lncRNA) and pcDNA3 vector (control), as previously described [3, 21]. In brief, PC-3 and DU 145 cells were nucleofected with plasmids (2 µg per 2 X 10<sup>6</sup> cells in 0.1 ml nucleofector solution V and L, respectively) using programmes T-013 and A-023, respectively, and cells were then cultured in 3 ml of the appropriate culture medium in 6-well plates for 24 h before use.

## **RNA interference by siRNA**

Trypsinized 22Rv1 and LNCaP cells were plated in 6-well plates (2 X 10<sup>5</sup>/well). At 30 min post-plating, cells were transfected with Ambion Silencer Select siRNAs to GAS5 using HiPerFect reagent and a fast-forward protocol, according to the manufacturer's instructions; the final siRNA concentration was 186 nM. Two different siRNAs were employed for GAS5 knockdown, termed GAS5#3 and #4, as previously described [10]. Controls were transfected with negative control (NC) siRNA. Transfection efficiencies, determined in parallel transfections with Cy3-labelled NC siRNA, were 97 ± 3% for 22Rv1 and 90 ± 5%

for LNCaP at 48 h post transfection. Cells were cultured for 24 h post-transfection before further experimentation.

#### **Assessment of mTOR inhibitor sensitivity.**

At 24 h post transfection, cells were plated in 96-well plates (500 cells in 0.1 ml medium) and also 6-well plates ( $2 \times 10^5$  cells in 2 ml medium) and incubated overnight. An equal volume of medium containing the appropriate drug or vehicle (final concentrations: 1  $\mu$ M rapamycin, 10 nM everolimus, 10 nM temsirolimus, 100 nM BEZ235, 50 nM AZD8055 or 0.1% DMSO) was then added [21]. Cells were harvested at 24 h (6-well plates: for GAS5 determination) or at 72 h (96-well plates: for MTS assay).

#### **Culture Proliferation assay**

Growth was assessed by a tetrazolium salt (MTS) assay or by direct microscopic counting. The MTS assay comprised CellTiter 96 Aqueous One Solution Cell Proliferation Assay, which was used in accordance to the manufacturer's instructions and as previously described [10, 21]. Medium blanks containing the drugs under test were run with each assay and the absorbance readings at 490 nm ( $A_{490}$ ) were subtracted from the appropriate test readings. For direct microscopic counting, adherent cells were collected by trypsinisation, stained with 0.1% [w/v] nigrosin blue, and counted using a hemocytometer; cells that excluded the dye were considered to be viable.

#### **Clonogenic Growth Assay**



Long-term survival of cells was determined by a colony forming assay. Cells were washed 48 h post drug treatment in sterile PBS and re-suspended in fresh medium supplemented with 10% cell-conditioned medium (1 mL/cm<sup>2</sup> culture area), and proportions (usually 2.5-10.0 µl cells) were plated into 6-well culture plates in 2.5 mL final culture volume. After 2-4 weeks, cells were stained with crystal violet (0.1% (w/v) in methanol) and the number of colonies (comprising ≥10 cells) were counted.

### **Quantitative RT-PCR**

Total RNA was extracted from cells, as previously described [10, 25, 26]. RNA was DNase treated prior to cDNA synthesis. cDNA was synthesized using 200 U of Moloney mouse leukaemia virus reverse transcriptase using Random hexamer Primer (Promega) according to the manufacturer's instructions and as previously described [25, 27].

The primer sequences used for GAS5 quantification were: forward 5'-CCTGGACCAGCTTAATGGTT-3' and reverse 5'-GTCTGCCTGCATTTCTTCAA-3' (product size 190 bp). Briefly, reactions (12 µl final volume) contained 1X Brilliant SYBR green QPCR mastermix, 400 nmol each of forward and reverse primers and 10 ng sample cDNA. All samples were analysed in triplicate [25, 26]. The target genes were normalised to endogenous controls GAPDH and or 18S rRNA, and relative quantification was carried out using the  $2^{-\Delta\Delta CT}$  method, where:  $-\Delta\Delta CT = \text{cycle threshold (CT)}_{(\text{GAS5 of cell line-GAPDH cell line})} - (\text{GAS5 of vehicle-GAPDH of vehicle})$ . Data are expressed relative to vehicle treated controls.

### **Statistical Analyses**

All experiments were repeated at least three times with triplicate determinations in each set of experiments. Data are presented as the mean  $\pm$  SEM; the number of observations (n) refers to number of different experiments, each derived from a separate culture. Data analysis was carried out by one-way ANOVA with Dunnett's multiple comparison post-test or Bonferroni's multiple comparison test using GraphPad Prism.

## Results

### **Rapamycin and rapalogues selectively modulate GAS5 expression and culture growth in prostate cell lines**

The effectiveness of mTOR inhibitors to enhance cellular GAS5 lncRNA levels in a range of prostate cell lines was examined, since GAS5 promotes the death of prostate cancer cells [10]. The first generation mTOR inhibitors rapamycin, everolimus and temsirolimus were chosen initially for this study [28-30]. Furthermore, a range of established, well characterized prostate cell lines were studied, comprising: PNT2C2, derived by immortalisation of normal prostate cells [31]; 22Rv1, an androgen-sensitive cell line derived from a prostate cancer xenograft [32]; LNCaP, an androgen-dependent cell line derived from a supraclavicular lymph node metastasis of prostate cancer [33]; PC-3, an androgen-independent cell line derived from a bone metastases of prostate cancer [34] and DU 145, an androgen independent cell line derived from a brain metastasis of prostate cancer [35].

In general, first generation mTOR inhibitors markedly enhanced GAS5 expression and slightly inhibited the growth of PNT2C2 cells (Fig. 1A). In the prostate cancer cell lines, 22Rv1 (Fig. 1B) and LNCaP (Fig. 1C), cellular GAS5 levels were enhanced by all agents to a similar extent as for PNT2C2 cells, but more potent inhibition of culture growth and clonogenic activity was seen. In contrast, PC-3 and DU 145

cells, were characterized by resistance to all inhibitors, in terms of GAS5 cellular levels, short-term culture growth and clonogenic activity (Fig. 1D and 1E).

### **Second generation mTOR inhibitors selectively modulate GAS5 expression and growth in prostate cell lines**

The mTOR inhibitors are in increasing clinical use, and resistance to such agents, especially first generation inhibitors which act selectively on mTORC1, is becoming progressively evident. This has prompted the development of inhibitors with broader specificity, i.e the dual mTORC1/mTORC2 inhibitor, AZD8055, and the combined PI3K/mTOR inhibitor, BEZ235. In related work, we have observed that these newer generation inhibitors can effectively enhance cellular GAS5 levels in breast cancer cells which otherwise show resistance to selective mTORC1 inhibitors [21]. For these reasons, we examined the effects of such agents on prostate cell lines.

However, the pattern of cell line response to both AZD8055 and BEZ235 was similar to that of the rapalogues, insofar as these two drugs elevated GAS5 expression and inhibited growth/clonogenic activity in PNT2C2 (Fig. 2A), 22Rv1 (Fig. 2B) and LNCaP cells (Fig. 2C) only, whereas the PC-3 and DU 145 cell lines showed no increase in GAS5 levels accompanied by complete resistance to these agents (Fig. 2D and 2E).

### **Endogenous GAS5 expression**

Previous work has shown that GAS5 levels are reduced in prostate cancer cell lines representative of advanced disease, relative to immortalized prostate cells [22]. Indeed measurement of GAS5 lncRNA levels in actively growing cells in the present study confirmed these findings. Thus, relative to PNT2C2

cells, values were similar in 22Rv1 cells but markedly lower in LNCaP, DU 145 and PC-3 cells (Fig. 3). Among the latter group of cell lines, which are all derived from prostate metastases, levels were significantly lower in PC-3 cells compared with LNCaP cells, whereas DU 145 cells exhibited intermediate levels of expression (Fig. 3).

#### **GAS5 silencing attenuates mTOR inhibitor action in prostate cancer cells.**

GAS5 lncRNA has been previously shown to be required for mTOR inhibitor action in a range of lymphoid cell lines [36], and a similar relationship may account for the reduced sensitivity of PC-3 and DU 145 cells to mTOR inhibitors, particularly since GAS5 levels are low in these cell lines. To test the postulate that GAS5 lncRNA is required for mTOR inhibitor action in prostate cancer cells, we initially examined the effect of GAS5 silencing on mTOR inhibitor action in the two sensitive cell lines, 22Rv1 and LNCaP, which exhibit relatively high and low steady state levels of GAS5 lncRNA, respectively (Fig. 4 and 5). Two different GAS5 siRNAs were employed in these experiments to reduce the likelihood of 'off-target' effects.

In 22Rv1 cells, GAS5 transcript levels were reduced by *ca.* 80% in cells treated with each GAS5 siRNA (Fig. 4A). This was accompanied by a small (*ca.* 30%) reduction in culture growth (Fig. 4B). Rapalogue treatment produced the expected growth inhibition in mock-transfected cells (Fig. 4C) and in cells transfected with negative control siRNA cells (Fig. 4D), whereas prior GAS5 silencing, markedly and consistently attenuated such growth inhibition (Figs. 4E and 4F).

The two GAS5 siRNAs also markedly reduced GAS5 expression in LNCaP cells (Fig. 5A), albeit this was without any effect on culture growth (Fig. 5B). In agreement with findings in 22Rv1 cells, rapalogues markedly inhibited the growth of control LNCaP cells (Figs. 5C and 5D) but had little effect on cells in which GAS5 expression had been silenced (Figs. 5E and 5F). Taken together, these findings support

the hypothesis that GAS5 lncRNA is required for the growth inhibitory activity of rapalogues in prostate cancer cells.

### **GAS5 lncRNA sensitizes PC-3 and DU 145 cells to mTOR inhibitors**

We next examined if prior transfection with GAS5 lncRNA could influence the sensitivity of PC-3 and DU 145 cells to both first generation and second generation mTOR inhibitors (Figs. 6 and 7). Nucleofection of the GAS5 plasmid construct enhanced cellular GAS5 levels three-fold in both PC-3 (Fig. 6A) and DU 145 cells (Fig. 7A) and this was without effect on the overall rate of culture growth (Figs. 6B and 7B). Both mock- and vector-transfected controls exhibited complete insensitivity to all mTOR inhibitors in terms of their culture growth (Figs. 6C, 6D, 7C and 7D), as expected, whereas cells transfected with the GAS5 lncRNA exhibited marked inhibition of culture growth upon rapalogue treatment (Figs. 6E and 7E). These findings therefore suggest that a minimum level of GAS5 lncRNA is required for the cytostatic/cytotoxic action of mTOR inhibitors on prostate cancer cells.

## **Discussion**

A major problem with current, first line androgen deprivation therapy for prostate cancer is the development of CRPC, which ultimately proves lethal [28]. Thus there is an urgent need to develop new therapies for CRPC and GAS5 lncRNA may offer new opportunities in this regard, since the survival of prostate cancer cells upon challenge with chemotherapeutic agents and other inducers of apoptosis has been shown to inversely correlate with cellular GAS5 lncRNA levels [10]. Here we have investigated mTOR inhibition as a strategy to enhance GAS5 levels in prostate cancer cells, and we demonstrate that such agents are effective in androgen-dependent/sensitive cell lines (22Rv1 and LNCaP) but not

in androgen-independent cell lines (PC-3 and DU 145). The latter cells are characterized by relatively low levels of endogenous GAS5 lncRNA, and we further demonstrate that GAS5 lncRNA is itself required for mTOR inhibitor action in prostate cancer cells, thereby providing an explanation for the resistance of androgen-independent cells to mTOR inhibition. Moreover, these findings have important implications for prostate cancer therapies involving mTOR inhibitors.

Currently there is much interest in the use of mTOR inhibitors as novel therapies in prostate cancer, and mTOR inhibitors would therefore appear an attractive pharmacological option to enhance cellular GAS5 lncRNA levels [16, 37]. First and second generation mTOR inhibitors exerted robust effects on both androgen-dependent (LNCaP) and androgen-sensitive (22Rv1) prostate cancer cell lines, whereas androgen-independent PC-3 and DU 145 cells failed to respond to these agents (at the concentrations studied here), both in terms of GAS5 expression and culture growth. Similar findings have been reported for breast cancer cell lines, insofar as growth and GAS5 expression in hormone-independent cells show greater resistance to mTORC1 and combined mTORC1/mTORC2 inhibition than hormone-sensitive cells [21]. Thus using mTOR inhibitors to elevate GAS5 levels in prostate cancer cells in order to promote their death is unlikely to succeed in CRPC, but it may be successful in less advanced disease. Alternative approaches are therefore required to elevate GAS5 expression levels in CRPC.

Studies in lymphoid cells have shown that GAS5 lncRNA is required for rapalogue action, but this issue has not been previously addressed in prostate cells or any other epithelial cell type [20]. Since endogenous GAS5 lncRNA levels are shown to be lower in prostate cancer cell lines derived from metastases than in immortalized prostate cells, in confirmation of previous findings [[22], we postulated that a similar relationship may account for mTOR sensitivity in prostate cells. Here we demonstrate that prior GAS5 silencing induces mTOR inhibitor-insensitivity (both in terms of GAS5

lncRNA expression and culture growth) in the otherwise mTOR-sensitive cell lines, 22Rv1 and LNCaP, thereby demonstrating a requirement for GAS5 lncRNA for rapalogue action. Moreover, upon transfection with GAS5 lncRNA, PC-3 and DU 145 cells acquire rapalogue sensitivity, further demonstrating that GAS5 lncRNA modulates mTOR inhibitor action. Thus decreased GAS5 expression in prostate cancer cells, such as occurs upon the acquisition of castrate-resistance, not only has the potential to attenuate the action of classical chemotherapeutic drugs [10], but also that of more novel targeted therapies, such as mTOR inhibitors. Better understanding of the mechanisms underlying reduced expression of GAS5 lncRNA upon the acquisition of castrate resistance is required, as this will help identify potential alternative means to enhance cellular GAS5 expression. Currently we are investigating epigenetic mechanisms in this respect, as well as the involvement of the recently discovered GAS5 antisense RNA 1 (*GAS5-AS1*) gene.

Current findings have broader implications for the use of mTOR inhibitors in prostate cancer. The oncogenic PI3K/Akt/mTOR signalling pathway plays a key role in the development and maintenance of prostate cancer and is deregulated in the majority of advanced prostate cancers [20]. In particular, the pathway serves as a critical nexus for the integration of growth signals with downstream cellular processes so that its activation such as occurs in prostate cancer [7-9] provides mechanisms for cancer cells to overcome the stress associated with, for example, androgen deprivation [5, 38]. These considerations have prompted the active investigation of mTOR inhibitors, either as single agents or in combination with other agents, as a novel targeted therapy for advanced prostate cancer, and the clinical effectiveness of single agent, first-generation mTOR inhibitors, such as everolimus and temsirolimus, has so far been disappointing. Our data, demonstrating an association between low GAS5 levels and increased resistance to the growth inhibitory effects of rapalogues in a model of androgen-independent disease, coupled with the direct demonstration that GAS5 lncRNA modulates mTOR inhibitor action in advanced prostate cancer cells, raises the possibility that low GAS5

expression in advanced prostate cancer may contribute to the clinical ineffectiveness of rapalogues in this disease. Current findings may also have broader implications for the clinical use of mTOR inhibitor-based therapies in a wide range of other cancers, including head and neck squamous cell carcinoma [39], glioblastoma multiforme [40], renal clear cell carcinoma [41], bladder cancer [42], pancreatic cancer [43] and non-small cell lung cancer [44], all of which are characterised by deficient GAS5 expression.

## Conclusions

Cellular GAS5 expression can be enhanced by the use of mTORC1/mTOR inhibitors in androgen-dependent/sensitive prostate cancer cells but not in androgen-independent cells. The refractoriness of the latter cells may in part be due to their low levels of endogenous GAS5 lncRNA expression, since GAS5 lncRNA positively regulates mTOR inhibitor action. Additional approaches are therefore required to increase GAS5 lncRNA expression in advanced prostate cancers in order to promote the effectiveness of chemotherapeutic agents and ultimately to improve and enhance patient outcomes.

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## Figure Legends

**Figure 1. Effects of pharmacological inhibitors of mTORC1 activity on GAS5 expression, culture growth and the survival of prostate cell lines.** Effects of rapamycin 1  $\mu$ M (R), everolimus 10 nM (E) and temsirolimus 10 nM (T) on GAS5 expression (24h; left-hand panel), culture growth (72 h; middle panel) and clonogenic survival (right-hand panel) in: **A** PNT2C2 cells **B** 22Rv1 cells, **C** LNCaP cells, **D** PC-3 cells and **E** DU 145 cells. Results are expressed as a proportion of the respective vehicle-treated controls. Each bar represents the mean  $\pm$  SEM from three independent experiments. Data were analysed by one-way ANOVA with Dunnett's multiple comparison post-test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  and \*\*\*,  $P < 0.001$  versus vehicle.

**Figure 2. Effects of combined mTORC1/mTORC2 and dual mTOR/PI3K inhibitors on GAS5 expression, culture growth and the survival of prostate cell lines.** Effects of AZD8055 (50 nM) and BEZ235 (100 nM) on GAS5 expression (24h; left-hand panel), culture growth (72h; middle panel) and clonogenic survival (right-hand panel) in: **A** PNT2C2 cells, **B** 22Rv1 cells, **C** LNCaP cells, **D** PC-3 cells and **E** DU 145 Cells. Results are expressed as a proportion of the respective vehicle-treated controls. Each bar represents the mean  $\pm$  SEM from three independent experiments. Data were analysed by one-way ANOVA with Dunnett's multiple comparison post-test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  and \*\*\*,  $P < 0.001$  versus vehicle.

**Figure 3. Endogenous levels of GAS5 lncRNA in prostate cell lines.** Endogenous GAS5 lncRNA levels were determined in actively growing prostate cell lines by RT-qPCR. Data are expressed as a percentage relative to PNT2C2 cells using  $\Delta$ CT analysis, using GAPDH for normalization. Each bar represents the mean  $\pm$  SEM for three independent experiments. Data were analysed by one-way ANOVA with Bonferroni's multiple comparison test. #,  $P < 0.001$  vs PNT2C2, \*,  $P < 0.001$  vs 22Rv1, ^,  $P < 0.001$  vs LNCaP, -,  $P < 0.05$  vs DU 145.

**Figure 4. Effect of GAS5 silencing on mTOR inhibitor action in 22Rv1 cells.** 22Rv1 cells were transfected with the indicated GAS5 siRNA (GAS5#3 or GAS5#4), negative control (NC) siRNA or mock-transfected and, after 48 h, samples were collected for determination of GAS5 lncRNA levels and cells were exposed to rapalogues (rapamycin 1  $\mu$ M (R), everolimus 10 nM (E) and temsirolimus 10 nM (T)) for 72 h. **A** GAS5 lncRNA levels; data are expressed relative to mock-transfected cells. **B** MTS assay results comparing the absolute growth of vehicle-treated, transfected cells. **C-F** MTS assay of the effect of rapalogues on the growth of transfected cells; data are expressed relative to respective vehicle (V)-treated controls for: **C** mock-transfected cells, **D** NC transfected cells, **E** GAS5#3 transfected cells and **F** GAS5#4 transfected cells. Each bar represents the mean value  $\pm$  SEM from three independent experiments. Data were analysed for significance by one-way ANOVA with Dunnett's multiple comparison post-test. \*,  $P < 0.05$  and \*\*\*,  $P < 0.001$  versus the respective vehicle-treated control.

**Figure 5. Effect of GAS5 silencing on mTOR inhibitor action in LNCaP cells.**

LNCaP cells were transfected with the indicated GAS5 siRNA (GAS5#3 or GAS5#4), negative control (NC) siRNA or mock-transfected and, after 48 h, samples were collected for determination of GAS5 lncRNA levels and cells were exposed to rapalogues (rapamycin 1  $\mu$ M (R), everolimus 10 nM (E) and temsirolimus 10 nM (T)) for 72 h. **A** GAS5 lncRNA levels; data are expressed relative to mock-transfected cells. **B** MTS assay results comparing the absolute growth of vehicle-treated, transfected cells. **C-F** MTS assay of the effect of rapalogues on the growth of transfected cells; data are expressed relative to respective vehicle (V)-treated controls for: **C** mock-transfected cells, **D** NC transfected cells, **E** GAS5#3 transfected cells and **F** GAS5#4 transfected cells. Each bar represents the mean value  $\pm$  SEM from three independent experiments. Data were analysed for significance by one-way ANOVA with Dunnett's multiple comparison post-test. \*,  $P < 0.05$  and \*\*\*,  $P < 0.001$  versus the respective vehicle-treated control.

**Figure 6. Effect of GAS5 lncRNA on mTOR inhibitor action in PC-3 cells.**

PC-3 cells were transfected with plasmids corresponding to mature lncRNA (pcDNA3+GAS5) or empty vector (pcDNA3), or mock-transfected. At 24 h, samples were taken for determination of GAS5 lncRNA levels, and cells were treated with mTOR inhibitors (rapamycin [1  $\mu$ M; R]; everolimus [10 nM; E]; temsirolimus [10 nM; T]; AZD8055 [50 nM; AZD]; and BEZ235 [100 nM; BEZ]) for 72h. **A** GAS5 lncRNA levels; data are relative to mock-transfected cells. **B** MTS assay results comparing the absolute growth of vehicle-treated, transfected cells only. **C-E** MTS assay results for mTOR inhibitor-treated cells; data are expressed relative to vehicle-treated cells for: **C** mock-transfected cells, **D** vector-transfected cells, **E** GAS5 lncRNA-transfected cells. Each bar represents the mean value  $\pm$  SEM from three independent experiments. Data were analysed for significance by one-way ANOVA with Dunnett's multiple comparison post-test. \*\*\*,  $P < 0.001$  versus vehicle control.



**Figure 7. Effect of GAS5 lncRNA on mTOR inhibitor action in DU 145 cells.** DU 145 cells were transfected with plasmids corresponding to mature lncRNA (pcDNA3+GAS5) or empty vector (pcDNA3), or mock-transfected. At 24 h, samples were taken for determination of GAS5 lncRNA levels, and cells were treated with mTOR inhibitors (rapamycin [1  $\mu$ M; R]; everolimus [10 nM; E]; temsirolimus [10 nM; T]; AZD8055 [50 nM; AZD]; and BEZ235 [100 nM; BEZ]) for 72h. **A** GAS5 lncRNA levels; data are relative to mock-transfected cells. **B** MTS assay results comparing the absolute growth of vehicle-treated, transfected cells only. **C-E** MTS assay results for mTOR inhibitor-treated cells; data are expressed relative to vehicle-treated cells for: **C** mock-transfected cells, **D** vector-transfected cells, **E** GAS5 lncRNA-transfected cells. Each bar represents the mean value  $\pm$  SEM from three independent experiments. Data were analysed for significance by one-way ANOVA with Dunnett's multiple comparison post-test. . \*\*\*,  $P < 0.001$  versus vehicle control.

Figure 1

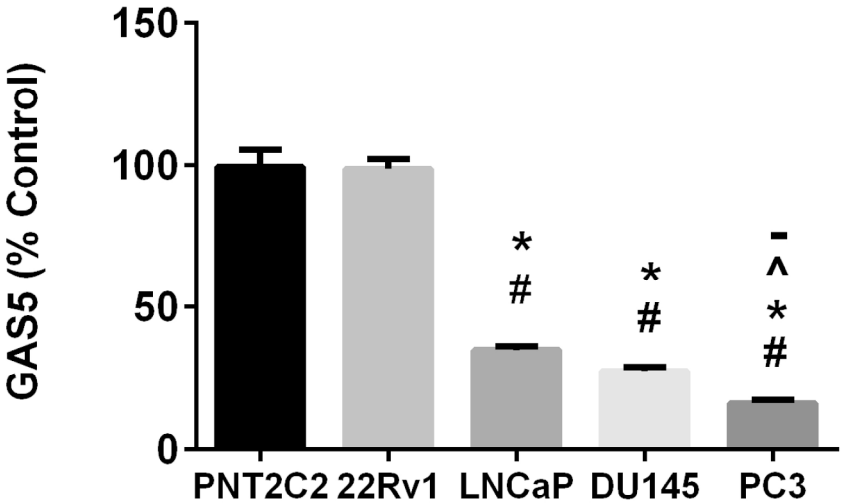


Figure 2

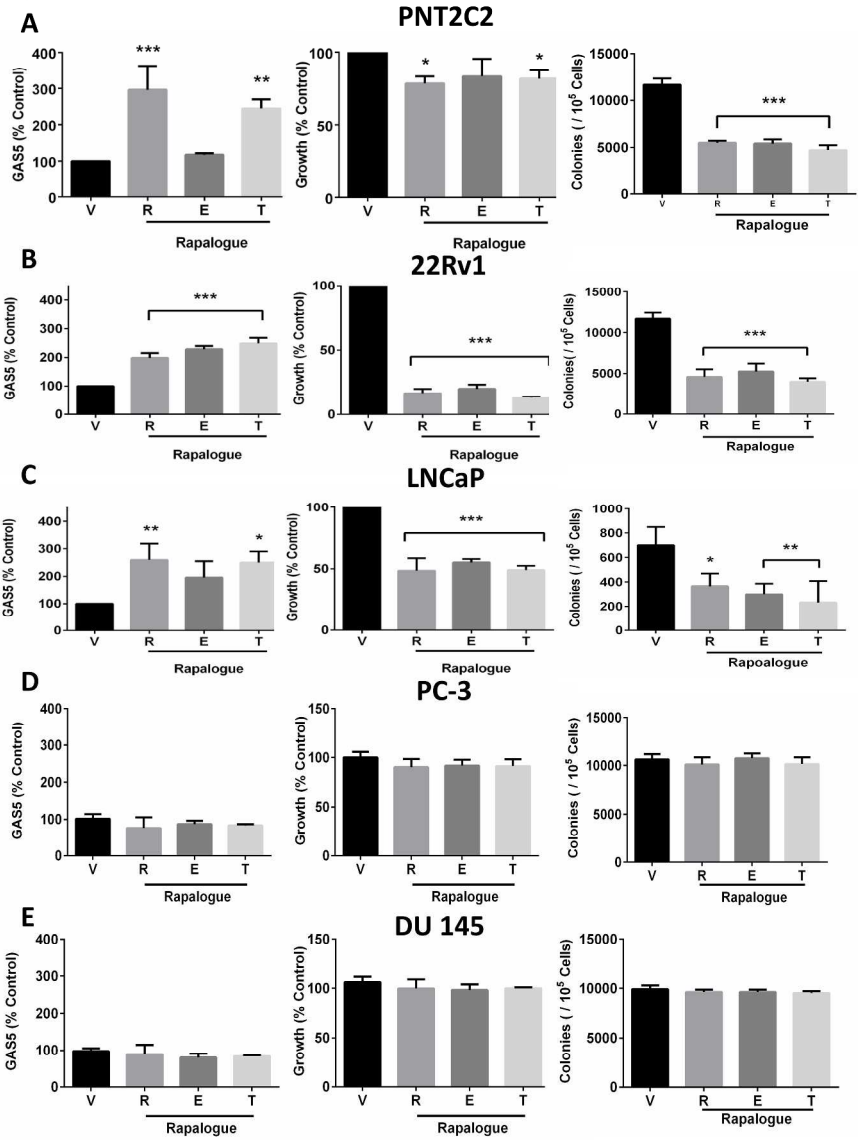
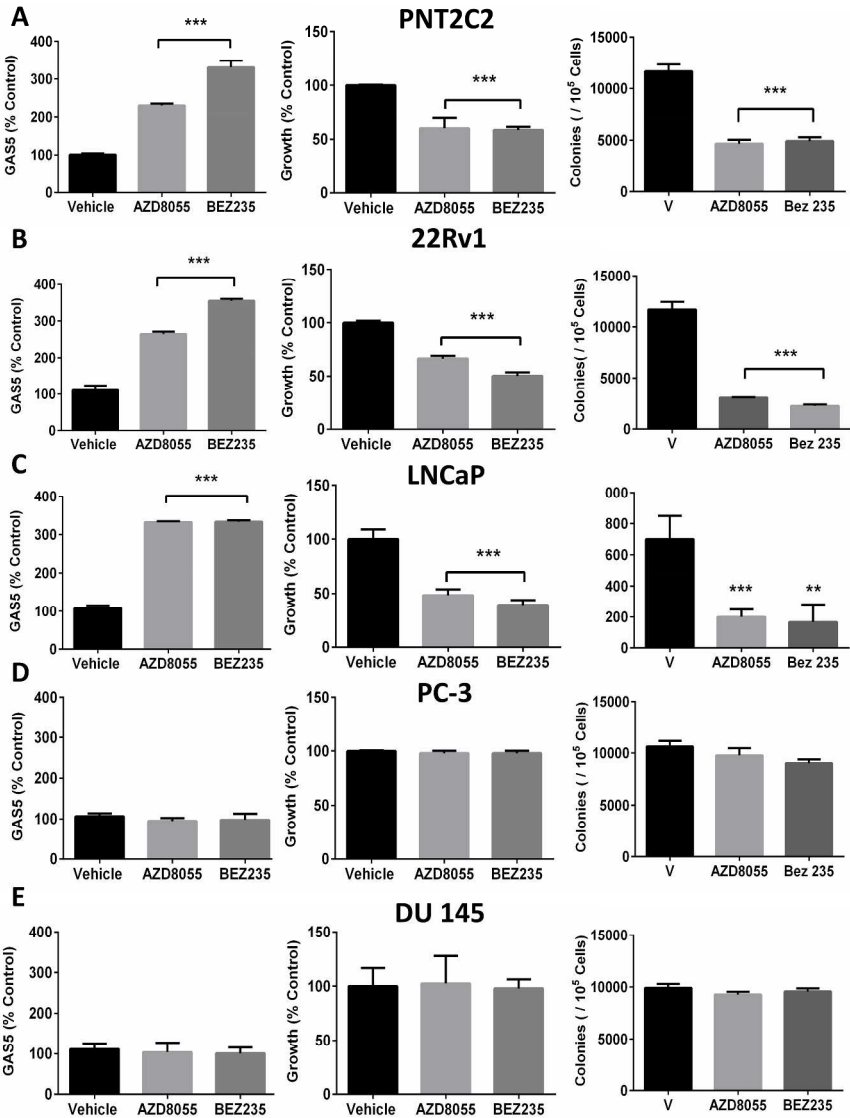
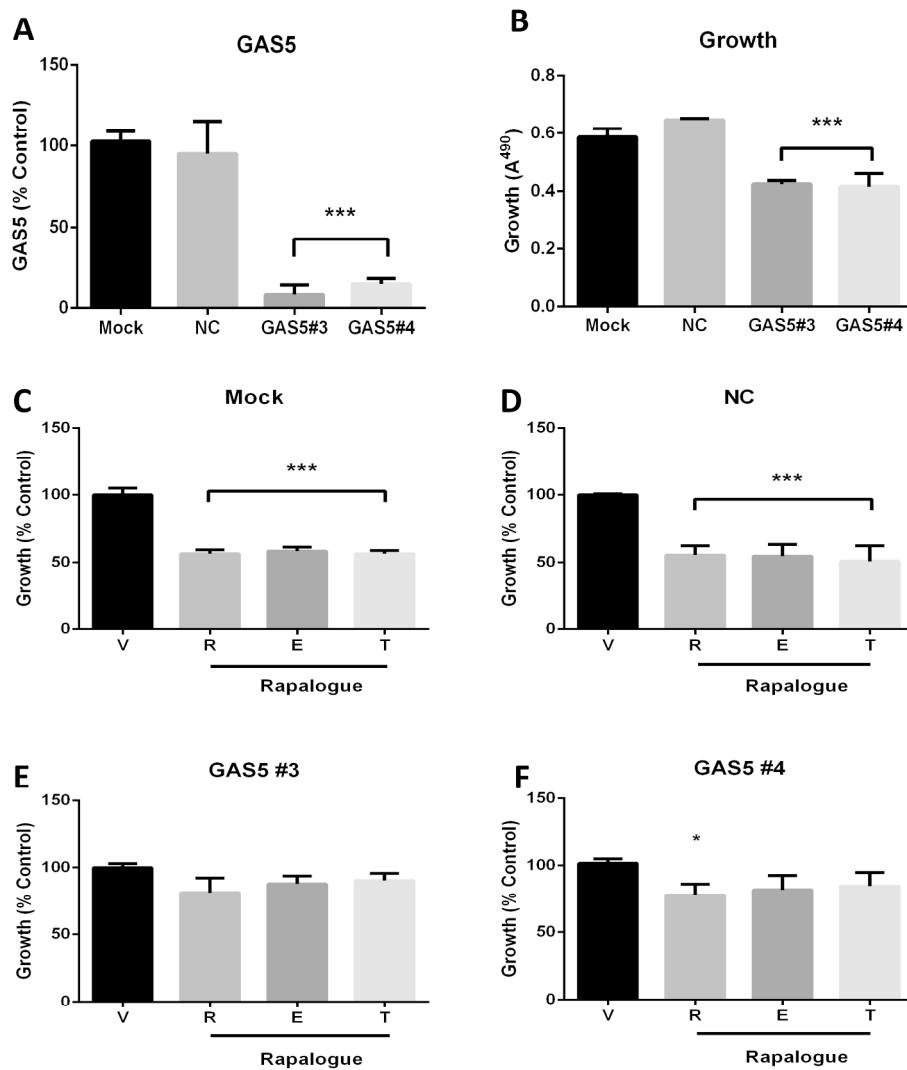


Figure 3



**Figure 4**



**Figure 5**

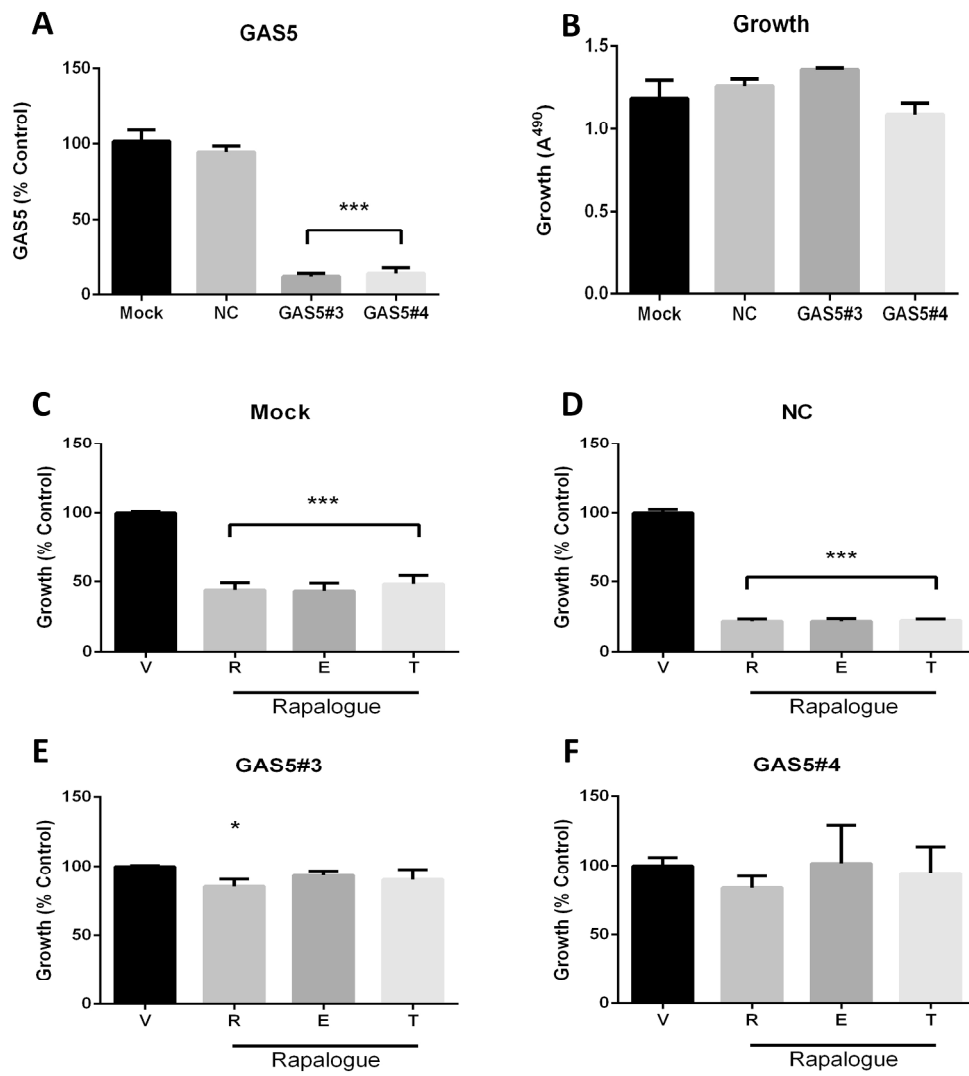


Figure 6

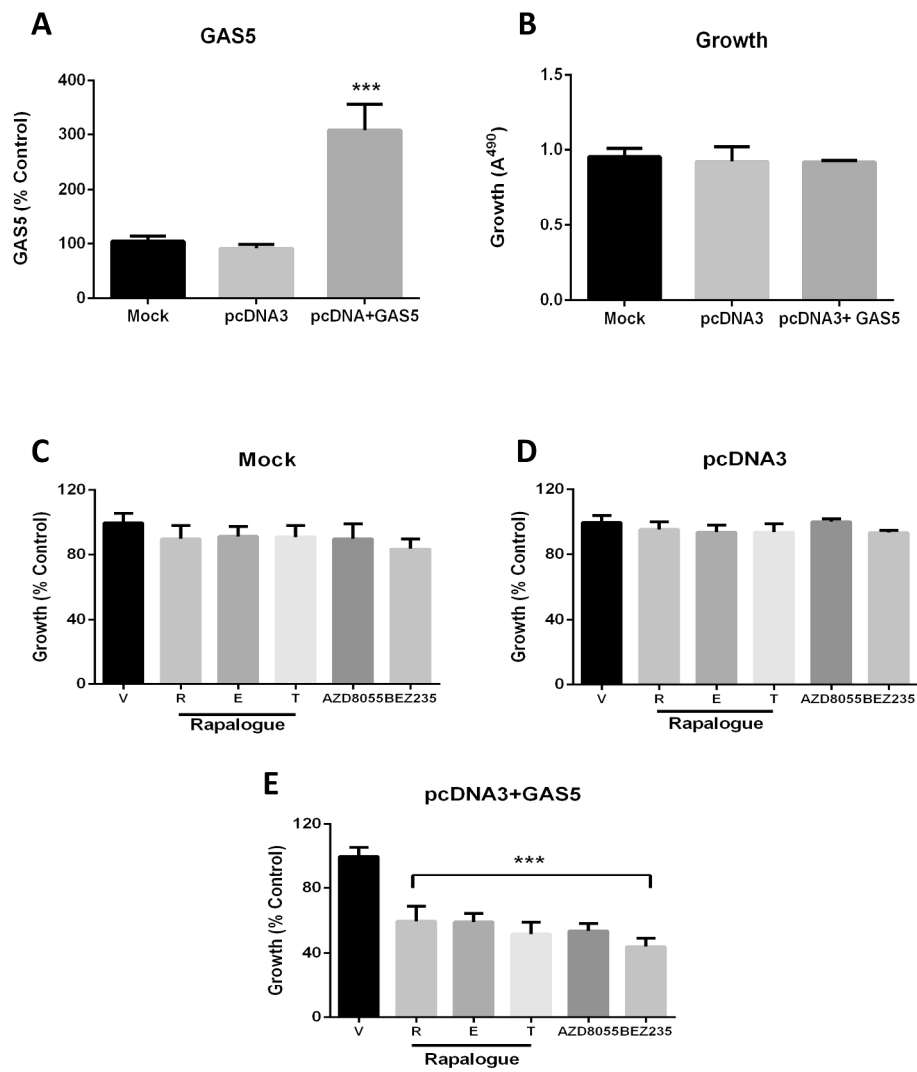


Figure 7

